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Abstract: Regulation of RANKL (receptor activator of nuclear factor B ligand)-induced osteoclast differentiation is of current interest in the development of antiresorptive agents. Osteoclasts are multinucleated cells that play a crucial role in bone resorption. In this study, we investigated the effects of N-methylpyrrolidone (NMP) on the regulation of RANKL-induced osteoclastogenesis. NMP inhibited RANKL-induced tartrate-resistant acid phosphatase activity and the formation of tartrate-resistant acid phosphatase-positive multinucleated cells. The RANKL-induced expression of NFATc1 (nuclear factor of activated T cells, cytoplasmic 1) and c-Fos, which are key transcription factors for osteoclastogenesis, was also reduced by treatment with NMP. Furthermore, NMP induced disruption of the actin rings and decreased the mRNAs of cathepsin K and MMP-9 (matrix metalloproteinase-9), both involved in bone resorption. Taken together, these results suggest that NMP inhibits osteoclast differentiation and attenuates bone resorption. Therefore, NMP could prove useful for the treatment of osteoporosis or other bone diseases associated with excessive bone resorption.

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INHIBITION OF OSTEOCLAST DIFFERENTIATION AND BONE RESORPTION BY N-METHYL PYRROLIDONE

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Running head: NMP reduces osteoclast differentiation and function.
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Regulation of receptor activator of nuclear factor κB-ligand (RANKL)-induced osteoclast differentiation is of current interest in the development of antiresorptive agents. Osteoclasts are multinucleated cells that play a crucial role in bone resorption. In the present study, we investigated the effects of N-methyl pyrrolidone (NMP) on the regulation of RANKL-induced osteoclastogenesis. NMP inhibited RANKL-induced tartrate-resistant acid phosphatase (TRAP) activity and the formation of TRAP-positive multinucleated cells (MNCs). The RANKL-induced expression of nuclear factor of activated T cells-c1 (NFATc1) and c-Fos, which are a key transcription factors for osteoclastogenesis, were also reduced by treatment with NMP. Furthermore, NMP induced disruption of the actin rings and decreased the mRNAs of Cathepsin K (catK) and Matrix metallopeptidase 9 (MMP9), both involved in bone resorption. Taken together, these results suggest that NMP inhibits osteoclast differentiation and attenuates bone resorption. Therefore, NMP could prove useful for the treatment of osteoporosis or other bone diseases associated with excessive bone resorption.

Introduction

Bone remodeling is a physiological process that involves the resorption and synthesis of bone by osteoclasts and osteoblasts, respectively (1,2). Osteoclasts are known to be formed by the fusion of hematopoietic cells of the monocyte-macrophage lineage during the early stage of the differentiation process (3). This process consists of multiple steps, including differentiation of osteoclast precursors into mononuclear osteoclasts, fusion of mononuclear preosteoclasts into mature multinucleated osteoclasts and activation of osteoclasts to resorb bone (4-7). The terminal differentiation in this lineage is characterized by acquisition of mature phenotypic markers such as expression of tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, matrix metalloproteinase 9, and cathepsin K, as well as morphological conversion into large multinucleated cells and the ability to form resorption lacunae on bone (8-10). The essential signaling molecules for osteoclast differentiation include RANKL and M-CSF (macrophage colony-stimulating factor) in bone marrow-derived macrophage precursor cells (11,12). RANKL is a member of the tumor necrosis factor (TNF) superfamily that is expressed in osteoblasts. It interacts with the osteoclast cell surface receptor RANK, which in turn recruits TNFR-associated factors (TRAFs) and plays a crucial role in the osteoclast differentiation axis (13,14). The downstream intracellular signaling mediated by RANK in osteoclast progenitor cells includes TRAF6-dependent activation of nuclear factor κB (NFκB) via the IκB kinase (IKK) complex and mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) (7,11,15). In addition, RANKL induces the key transcription factors for osteoclastogenesis, NFATc1 and c-Fos (9,16,17). Therefore, chemical or natural compounds that specifically inhibit these steps could be developed as antiresorptive drugs for the treatment of metabolic bone disorders characterized by excessive osteoclastic bone resorption.

Recently we found that NMP is a bioactive drug, since it enhances bone regeneration *in vivo* in rabbit calvarial defect model (18). At the cellular level, the pharmaceutical effect of NMP was confirmed since NMP increased early and late markers for maturation of preosteoblasts and human bone marrow-derived stem cells in vitro (18).

In the present study, we investigated the effects of NMP on RANKL-induced osteoclast differentiation in vitro, and characterized the role of NMP in osteoclast differentiation. We provide the first evidence that NMP inhibits RANKL-stimulated osteoclastogenesis by suppressing NFATc1 expression, and RANKL-induced osteoclasts function by disturbing actin rings formation and decreasing MMP 9 activity.

EXPERIMENTAL PROCEDURE

Reagents and antibodies. Recombinant RANK Ligand was purchased from Invitrogen (Life Technologies). NFATc1 (H-110) and c-Fos (H-125) polyclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polyclonal anti-ERK1/2, anti-pERK1/2 were obtained from Cell signaling (Cell signaling Technology, MA, USA). Trans-AM AP-1/c-Fos transcription factor kit was from Active Motif (Rixensart, Belgium). RNA extraction kit (RNeasy kit) was from QIAGEN. The BCA kit for protein determination was from Pierce Chemical Co. (Rockford, IL). TRAP solution was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were available from Sigma Chemical Co.

Cell cultures. RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin). The cultures were never allowed to become confluent. Incubations were performed at 37°C in 5% CO_2 in humidified air. Bone Marrow-Derived Macrophages (BMMs) were isolated from long bones of 6-week-old mice and were maintained in α-minimal essential medium (α-MEM) containing 10% heat-inactivated FBS in the presence of M-CSF (100 ng/ml) as described in (19). To generate osteoclasts from BMMs, cells were plated in 24-well tissue culture plates and cultured in the presence of 25 ng/ml RANKL and 25 ng/ml M-CSF.

Cell viability and proliferation assay. Effect of different concentrations of NMP on RAW264.7 cells proliferation / viability was analyzed using a non-radioactive WST-1 Cell Proliferation Assay kit (Roche Diagnostics) according to the manufacturer's instruction.

Tartrate resistant acid phosphatase (TRAP) activity and TRAP-staining. RAW264.7 cells were plated into a 12-well culture dish (Corning, NY) with different concentrations of NMP in the presence of 25 ng/ml of RANKL. The medium and factors were replaced every 2 days. After 6 days of culture, the medium was removed and the cell monolayer was gently washed twice with PBS. The cells were then lysed with 200 µl of 0.1% Triton X-100. TRAP activity in cell lysate was determined using TRAP solution (sodium acetate 0.1M pH 5.8, ascorbic acid 1 mM, KCl 0.15 M, disodium tartrate 10 mM and p-nitrophenyl phosphate 10 mM). An aliquot of cell lysate was added to TRAP solution and subsequently incubated for 30 minutes at 37°C. The reaction was stopped with NaOH 0.3 N and absorbance was measured at 405 nm using a micro-plate reader (BioTek). Results, normalized to protein content, are expressed in percentage of the activity obtained in RANKL stimulated cells. TRAP histochemical staining of the cells was performed using a leukocyte acid phosphatase kit (Sigma-Aldrich). Cultured cells were fixed with formaldehyde for 5 min at room temperature, washed with PBS and air-dried. After TRAP-staining, TRAP-positive multinucleated cells (more than 3 nuclei) were counted under phase-contrast microscopy.

Pit formation assay. RAW264.7 cells were placed on bone slices in 24-well plates. After preincubation for 6 h, the bone slices were transferred to 12-well plates (1 bone slice/well) and further cultured in the presence or absence of 25 ng/ml RANKL and 5mM NMP. The medium was replaced with a fresh one every 2 days. After 9 days of culture, cells were removed and pit area was visualized by toluidine blue staining and quantified by image analysis system. Resorption pits of representative cultures are shown.

Actin ring formation assay. Actin rings of osteoclasts were detected by staining actin filaments with rhodamine-conjugated phalloidin. Osteoclasts were formed from RAW264.7 cell cultures in the presence of RANKL (25 ng/mL) and NMP (1, 5 and 10 mM). At the end of incubation, osteoclasts were stained with rhodamin-conjugated phalloidin for actin and DAPI for nucleus. The distribution of actin rings was visualized and detected under a fluorescence microscope.

Quantitative Real-time RT-PCR. RNA from RAW264.7 cells was extracted using the RNeasy kit from Qiagen. The mRNA was reverse

transcribed into cDNA. The resultant cDNA was subjected to real-time PCR with gene-specific primers using iQ SYBR Green super-mix and iCycler real-time PCR machine (both from Bio-Rad Laboratories) according to the manufacturer's instructions. The primer sequences are presented in **table 1**.

Protein preparation and Western blot analysis. RAW264.7 cells treated with the different compounds were rapidly frozen in liquid nitrogen and stored at −80°C for further analysis. Cells were lysed as described in (20). Proteins were fractionated onto a 12% SDS polyacrylamide gel electrophoresis, transferred to Immobilon P membranes (Millipore, Switzerland), and immunoblotted with specific antibodies. Detection was performed using peroxidase-coupled secondary antibody, enhanced chemiluminescence reaction (Amersham ECL Western Blotting Detection Reagents, GE Healthcare Europe GmbH, Otelfingen, Switzerland), and visualization using autoradiography. Membranes that were reprobed had been stripped in stripping buffer (62.5 mM Tris-hydrochloric acid, pH 6.8, 2% (w/v) SDS, 100 mM β-mercaptoethanol) according to the manufacturer's protocol (Millipore).

Zymography. Equal volumes of conditioned medium were loaded onto a 10% SDS-PAGE gel containing 0.1% porcine gelatin (Sigma). After electrophoresis, the gels were washed twice for 15 min each in 2.5% Triton X-100 and then incubated overnight at 37 °C in substrate buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$ and 0.02% Brij 35). The gels were stained with Coomassie Blue R-250 (Sigma) for 1 h. The gels were de-stained briefly in 50% methanol, 10% acetic acid. Areas of gelatinolytic activity appeared cleared against the blue background of the blue-stained undigested gelatin.

AP-1 activation analysis. To detect AP-1 activation in RAW264.7 cells, we used ELISA-based Trans-AM AP-1 transcription factor kit (Active Motif, Rixensart, Belgium). Cells were plated in Petri dishes for 48h. Cells were pre-incubated 4h in serum-free fresh medium and stimulated for 30 minutes as indicated in the figure. Preparation of nuclear cell extract was done according to the manufacturer's instructions. The active form of c-fos in nuclear extracts can be detected using an antibody specific for an epitope that is accessible only when the nuclear factor is activated and bound to its target DNA. By using an antibody directed against c-Fos, the AP-1 dimer bound to the oligonucleotide is detected. A secondary antibody conjugated to horseradish peroxidase provides colorimetric visualisation. Absorbance was determined with a microplate reader (SynergyHT, BioTek).

Statistical analysis. Experiments were carried out independently at least three times. Results are expressed as the mean ± SD and were compared by Student's *t*-test. Results were considered significantly different for $p<0.05$.

RESULTS

NMP suppressed RANKL-induced osteoclastogenesis. To clarify the effects of NMP on osteoclastogenesis, we used RAW264.7 cells and bone marrow-derived macrophages (BMMs) (**Figure 1**). Cells were incubated with NMP in the presence of RANKL for RAW264.7 cells (**Figure 1a**) or NMP in the presence of RANKL and M-CSF for BMMs cells (**Figure 1b**) as indicated in the figure. In the absence of NMP, RAW264.7 cells differentiate into mature TRAP-positive multinucleated osteoclasts (MNCs), while NMP (5 and 10 mM) reduced the formation and numbers of TRAP-positive MNCs in a concentration-dependent manner (**Figure 1a**). The number of MNCs was quantified microscopically. In the presence of NMP (5 mM), the MNCs number induced by RANKL was reduced approximately by 50%, whereas 10 mM NMP abolished completely the formation of MNCs. In BMMs cells, NMP appears to have the same effect (**Figure 1b**). Indeed, treatment of BMMs with RANKL + M-CSF induces the formation of TRAP (+) MNCs and increases TRAP acitivity. NMP treatment completely blocked this effect. To exclude the possibility that the inhibition was due to cytotoxicity of NMP, cell cytotoxicity/viability was analyzed using a non-radioactive WST-1 Assay (**Figure 2**). NMP demonstrated no cytotoxic effects after 24 and 72 hours treatment with the concentration which effectively inhibited osteoclast formation by 50% (5 mM). We next examined in more details the effect of NMP on TRAP activity induced by RANKL in RAW 264.7 cells (**Figure 3**). We found that TRAP activity was significantly reduced in the cells treated with RANKL and NMP compared to the cells treated with RANKL alone (**Figure 3a**). Furthermore, NMP dramatically reduced the number of nuclei

per osteoclast suggesting that NMP could modulate the fusion process (**Figure 3b**).

NMP inhibits bone resorption and actin ring formation. We further examined if NMP has an effect on the ability of mature osteoclasts to resorb bone. RAW264.7 cells were plated on bone slices and stimulated with RANKL in the presence or absence of NMP. RANKL-stimulated cells formed a number of pits, suggesting that the bone resorption activity of RANKL-treated cells made them into functionally active state resembling osteoclasts (**Figure 4**). Treatment with 5 mM NMP significantly reduced the formation of resorption pits in numbers and in overall area as compared to treatment with RANKL alone. Bone resorption occurs within the sealing zone, which is formed by an actin ring structure. In order to investigate the effect of NMP on actin ring formation, immunofluorescence analysis was performed (**Figure 5**). The majority of RANKL treated cells revealed well-formed actin rings **(Figure 5a).** Cells treated with RANKL in the presence of 5 mM NMP displayed mainly disrupted actin rings (**Figure 5b**). As expected, cells treated with RANKL in the presence of 10 mM NMP shows no actin rings compared to cells treated with RANKL alone. Wilson et al., (21) reported that osteoclasts displaying a full actin ring or disrupted actin rings with more than 50% intact were identified as active. Our results are in line with this observation since with 1mM NMP, concentration which is ineffectively inhibited osteoclast differentiation, the actin ring is not completely disrupted **(Figure 5b).**

NMP suppressed RANKL-induced MMP-9 and Cathepsin K. The bone resorption-related enzymes MMP-9 and Cathepsin K, are highly expressed in osteoclastic cells and play an important role in skeletal remodelling (22,23). Therefore we investigated the effect of NMP on the expression of MMP-9 and Cathepsin K expression (**Figure 6**) in detail. First we evaluate MMP 9 activity by gelatine zymography (**Figure 6a**). RAW264.7 cells treated with RANKL showed a concentration-dependant increase in MMP-9 gelatinolytic activity. This gelatinolytic activity was significantly decreased by NMP treatment and was correlated with the suppression of osteoclast differentiation visualized when the same culture were stained for TRAP (data not shown). **Figure 6b** demonstrates that RANKL induced an increase in MMP-9 mRNA and only treatment with 10 mM NMP was able to decrease the RANKL-induced MMP-9 mRNA. Cathepsin K mRNA expression was also increased by RANKL treatment. In the presence of NMP (5 and 10 mM), RANKL-induced Cathepsin K mRNA was significantly suppressed (**Figure 6b**) already at 5mM NMP.

NMP inhibits NFATc1 and c-Fos expression and decreases AP-1 activation. NFATc1, a member of the NFAT family of transcription factor, has been shown to be up-regulated after RANKL stimulation and is important for osteoclast differentiation (10,16). Therefore, we examined the effect of NMP on the expression of NFATc1 **(Figure 7)**. The stimulation of RAW264.7 cells with RANKL induced a high level expression of NFATc1 mRNA (**Figure 7a**). The treatment with NMP (5 mM) significantly decreased RANKL-induced NFATc1 mRNA. RANKL-induced NFATc1 mRNA was completely blocked by 10 mM NMP treatment. As shown in **Figure 7b**, cellular protein expression was correlated with mRNA expression. Indeed, the RANKL-induced NFATc1 expression was significantly and concentration-dependently attenuated by NMP treatment. With 10 mM NMP, the expression of NFATc1 induced by RANKL becomes similar to untreated cells.

It is well known that c-Fos, a transcription factor for AP-1 complex, is required for osteoclast differentiation. This factor has been shown to bind to NFATc1 promoter and to be important for its activation (17,24). As shown in **figure 8a**, c-Fos expression was increased by RANKL. NMP treatment (5mM) significantly decreases c-Fos expression induced by RANKL. Next, we examined whether NMP could suppress AP-1 activity by TransAM AP-1 assays (**figure 8b**). The results showed that the AP-1 transcriptional activity was increased when cells were exposed to RANKL, whereas NMP suppressed AP-1 activity, suggesting that NMP can inhibit RANKL-induced AP-1 activation. The specificity of the assay was confirmed by using positive control and wild-type and mutant oligonucleotids (data not shown).

NMP inhibits RANKL-induced ERK pathway activation. Growth factors that stimulate ERK may ultimately control the activity of c-Fos and AP-1 dependent transcription (25). Recently, it has been reported that macrophage inflammatory protein (MIP)-1α induces osteoclast formation by activating the MEK/ERK/c-Fos pathway (26),

and vitamin E inhibited RANKL-induced osteoclast differentiation from precursors by suppression of c-Fos expression, possibly through inhibiting ERK (27). To investigate the role of ERK, JNK and p38 pathways in the inhibitory effect of NMP, RAW264.7 cells were treated as indicated in **figure 9a**, and whole cell extract was subjected to western-blot using phospho-ERK pathway antibodies. RANKL treatment induces ERK1/2 phosphorylation, and the presence of NMP resulted in a decrease in ERK1/2 phosphorylation. We also examined signaling steps upstream and downstream of ERK1/2. As shown in **figure 9a**, NMP suppressed the RANKL-induced MEK1/2 phosphorylation, but also the phosphorylation of two downstream targets of ERK1/2, p90RSK and MSK1. NMP reduced RANKL-induced ERK activation but not p38 and JNK, suggesting that the effect of NMP on ERK pathway is specific (**figure 9b**).

DISCUSSION

The major modalities currently used in osteoporosis treatment primarily include estrogen replacement therapy along with bisphosphonates, selective estrogen receptor modulators (SERM), and calcitonin. However, such therapies are associated with adverse effects, including breast cancer, hypercalcemia, and hypertension (5,28-32).

In medicine, N-methyl pyrrolidone (NMP) has a long track record as a constituent in medical devices approved by the Food and Drug Administration and thus can be considered as a safe and biologically inactive small chemical. Recently, we revealed that NMP enhances BMP activity by increasing the kinase activity of the BMP receptor complex for Smad 1,5,8 and p38 and could be employed as a potent drug for bone tissue regeneration and engineering (18). Bone remodeling is tightly regulated by two processes: bone formation and bone resorption. The balance between both processes is a key for maintaining bone density. In this study, we report that NMP, in addition to its role in bone formation, is also able to inhibit osteoclast differentiation in vitro, since NMP attenuates the formation of TRAP (+) MNCs from precursor cells stimulated with RANKL. Our results suggest that NMP inhibits MNCs formation, including fusion of the mononuclear precursor cells since it decreases the number of nuclei per MNCs. The number of nuclei per MNC should reflect the relative rate of fusion of mononuclear precursors to form MNCs.

NFATc1 and c-Fos are crucial transcription factors in RANKL-induced osteoclastogenesis. The role of c-Fos transcription factor in osteoclastogenesis has been revealed by knockout experiments (33). C-Fos knockout mice exhibited serious osteopetrotic phenotype, due to the failure of osteoclast formation. In addition, previous reports demonstrated that NFATc1 was not induced by RANKL stimulation in osteoclasts lacking c-Fos. The importance of NFATc1 on osteoclastogenesis was supported by *in vitro* experiments where NFATc1 knockout mice-derived stem cells failed to differentiate into osteoclasts and where bone marrow-derived macrophage cells, when forced to express NFATc1, differentiated to osteoclasts even in the absence of RANKL (24,33). In our study, NMP significantly suppressed NFATc1 up-regulation normally seen after RANKL treatment. Previous studies have shown that NFATc1, the master regulator of osteoclastogenesis, is regulated by AP-1 complex. AP-1 is a dimeric transcription factor composed of members of Jun and Fos family proteins. AP-1 converts extracellular signals in bone and immune cells into changes in the expression of specific target genes, which harbour an AP-1 binding site(s) in their promoter or enhancer regions (34).

Since c-Fos upregulation is needed for NFATc1 induction, it is possible that the suppression of NFATc1 expression by NMP is the consequence of the down-regulation of c-Fos, with subsequent down-regulation of AP-1 activity and inhibition of osteoclast-specific gene expression normally required for efficient osteoclast differentiation and bone resorption. This finding is important since AP-1 transcription factor links inflammatory processes to the activation of signalling pathways in osteoclast and therefore can be considered an important contributor to inflammatory bone diseases.

Active osteoclasts display characteristic membranes, including the ruffled border, attachment zone, and the basolateral secretory membrane. After attachment to bone, the ruffled border secretes enzymes and protons enabling the solubilization and digestion of the bone matrix. Rapid cytoskeletal reorganization is essential for osteoclast function and formation of the specialized membranes (21,35). The NMP treatment disrupted actin ring formation in

osteoclasts. More importantly, osteoclastic bone resorption was strongly inhibited by NMP. Moreover, NMP inhibited RANKL-induced MMP 9 and cathepsin K upregulation, both highly expressed in osteoclastic cells and playing an important role in skeletal remodeling. Recently, Wilson et al., (21) reported that cathepsin K activity is required for the initial formation of actin rings and thus for the activation of the osteoclasts. Our results are in line with this observation since at 5 and 10 mM NMP, concentrations which effectively suppressed RANKL-induced cathepsin K mRNA production and inhibited osteoclast differentiation, the actin rings are completely disrupted. Together, our results indicate that NMP plays a critical role in osteoclastic bone resorption by regulating actin ring formation and decreasing bone resorption-related enzymes.
MAPK signalling cascades regulate transcription by multiple mechanisms, including the phosphorylation and activation of transcription factors, coactivators, corepressors, and the basal transcriptional machinery. Activated ERK1/2 and/or p90RSK have been shown to target c-Fos (36-38) as well as participate in the activation of NF-κB (39). In this study, we reported that NMP decrease RANKL-induced activation of ERK pathway, and this inhibition might significantly reduces the induction of c-Fos trancription factor and subsequently AP-1 activation by RANKL.

In the present study, we demonstrated for the first time the inhibitory effect of NMP on osteoclast differentiation and function. NMP inhibits the RANKL-induced osteoclast differentiation and function. Accordingly, a model is proposed to explain the bioactivity of NMP (**Figure 10**). NMP might act as inhibitor of several key events. Particularly, NMP inhibits RANKL-induced ERK 42/44 phosphorylation which is important for c-Fos expression and AP-1 activation. Thus, the expression of NFATc1, which is regulated by AP-1, is decreased by NMP treatment. NMP is also able to inhibit osteoclast function by disturbing actin ring formation and reducing MMP 9 and cathepsin K expression, both involved in bone digestion. Therefore, NMP inhibits osteoclast differentiation by modulating AP-1 and NFATc1 transcription factors, and inhibits osteoclast function by reducing MMP-9 and Cathepsin K expression and by disrupting actin ring formation.

Taken together, our results demonstrate that NMP has an inhibitory activity on both osteoclast differentiation and function through mechanisms involving inhibition of the RANKL-induced AP-1 activation. But additional work is needed to further pinpoint the exact site(s) where NMP exerts its suppressive activity on osteoclasts.

Molecules, like NMP that enhance bone regeneration on one hand and suppress osteoclast differentiation on the other hand may have great therapeutic value in treating osteoporosis and other bone erosive diseases such as rheumatoid arthritis or metastasis associated with bone loss. Our findings suggest that NMP may potentially be useful for the treatment of bone diseases associated with excessive bone resorption.

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FOOTNOTES:

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The abbreviations used are: NMP, N-methyl pyrrolidone; RANKL, receptor activator of nuclear factor κB-ligand; TRAP, tartrate-resistant acid phosphatase; MNC, multinucleated cells; BMM, Bone Marrow-Derived Macrophages; NFATc1, nuclear factor of activated T cells-c1; MMP-9, matrix metalloproteinase-9; catK, cathepsin K; mOC, mature osteoclasts; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factors; M-CSF, macrophage colony-stimulating factor; AP-1, activator protein 1; NFκB, nuclear factor κB; IKK, IκB kinase complex, MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase, JNK, c-Jun N-terminal kinase; DMEM, Dulbecco's modified Eagle's medium; MNC, multinucleated osteoclasts.

FIGURE LEGENDS

Gene	Sequence (5' -> 3')	Amplicon Size
GAPDH	**Forward** AGGTCGGTGTGAACGGATTTG **Reverse** TGTAGACCATGTAGTTGAGGTCA	123
NFATc1	**forward** GGAGCGGAGAAACTTTGCG **Reverse** GTGACACTAGGGGACACATAACT	94
c-Fos	**Forward** CGGGTTTCAACGCCGACTA **Reverse** TTGGCACTAGAGACGGACAGA	166
MMP 9	**Forward** CTGGACAGCCAGACACTAAAG **Reverse** CTCGCGGCAAGTCTTCAGAG	145
Cathepsin K	**Forward** GAAGAAGACTCACCAGAAGCAG **Reverse** TCCAGGTTATGGGCAGAGATT	102

Table 1. *Primer sequences used in real-time PCR*

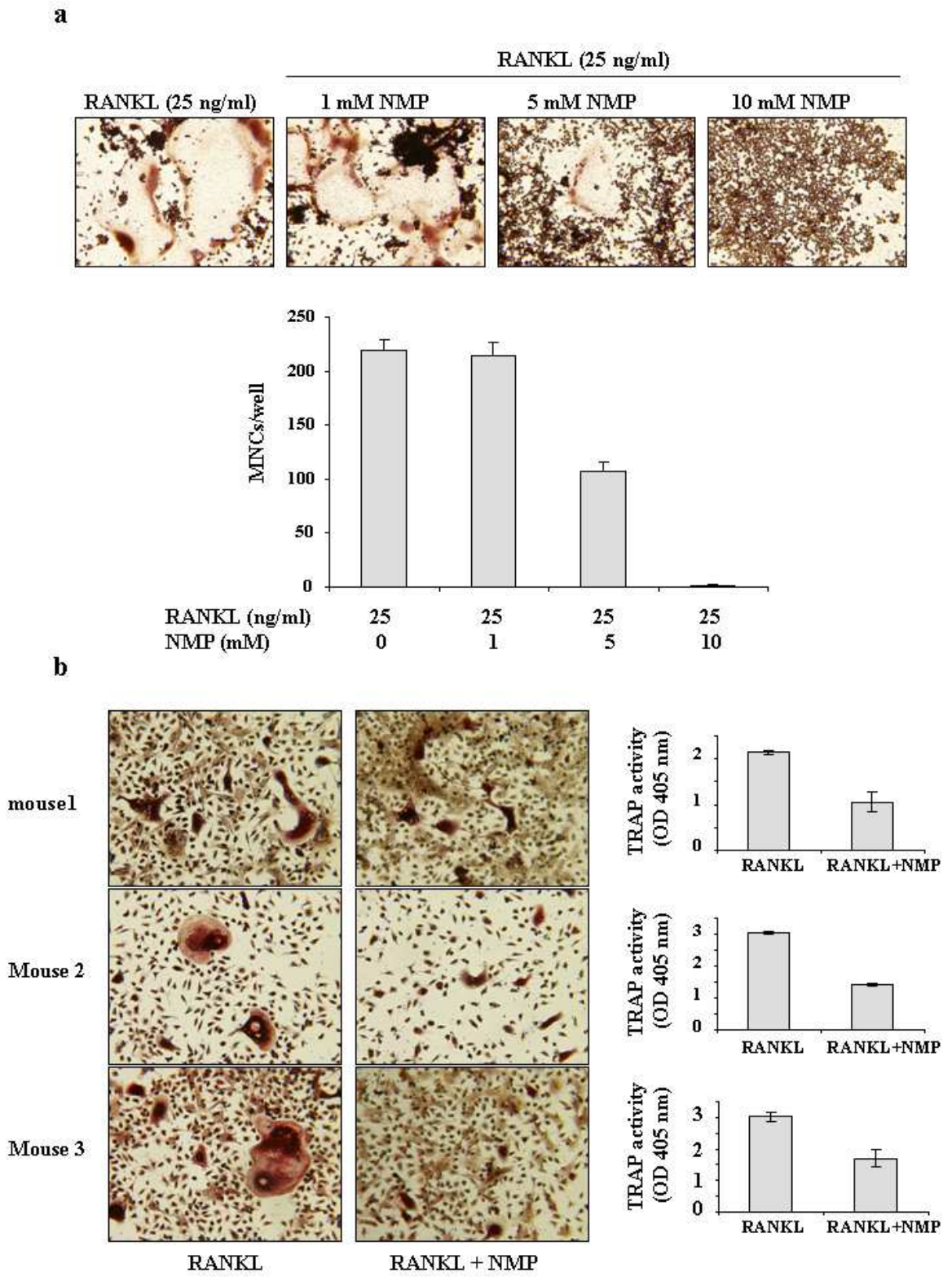

Figure 1. *Effects of NMP on osteoclastogenesis.* (**a**) RAW264.7 cells were seeded in 12-well culture plates and treated for 6 days with NMP, RANKL or NMP+RANKL as indicated in the figure. For the visualisation of TRAP-positive multinucleated cells (MNCs), the cells were fixed and stained for TRAP activity as described in Methods. Stained cells were photographed, and TRAP (+) MNCs containing three or more nuclei were counted as osteoclasts. Data are expressed as mean S.D. (n=3). (**b**) BMMs from three different mice were treated with NMP (5 mM) in the presence of M-CSF (25 ng/ml) and RANKL (25 ng/ml) for 4 days. After culturing, the cells were fixed and stained for TRAP. In parallel, TRAP activity was determined as described in Methods. Data are expressed as mean S.D. (n=4) from a representative experiment.

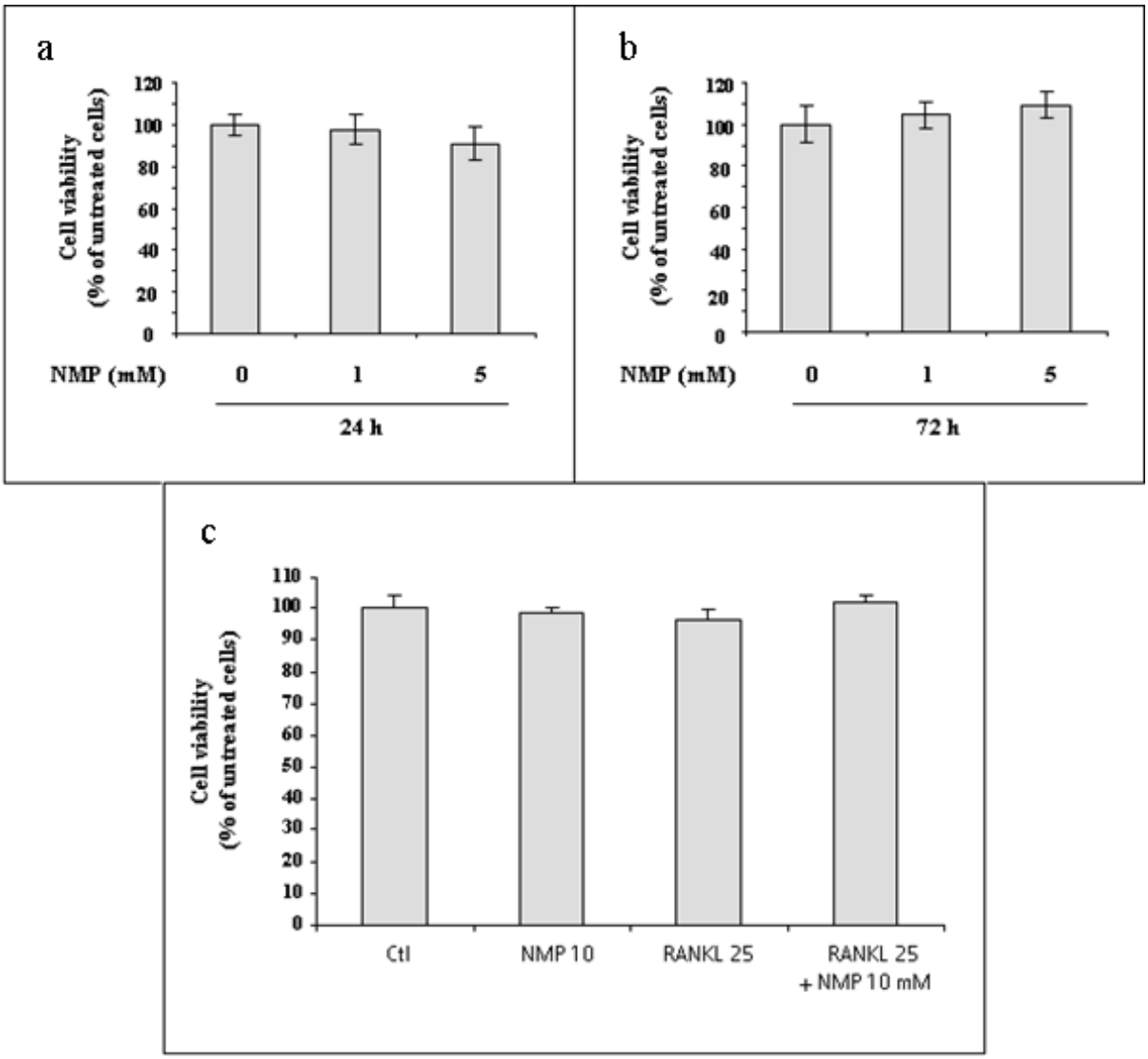

Figure 2. *NMP had no cytotoxic effect on Raw 264.79 cells*. Cells were seeded on a 96-well plate, treated for either 24h (**a**) or 72h (**b**) with different concentration of NMP, or treated with RANKL and NMP as indicated in the figure (**c**). Cell viability/cytotoxicity was measured using WST-1 reagent as described in Methods. The results were normalized to cells grown in DMEM alone (Ctl). Data are expressed as mean S.D. (n=4) from a representative experiment.

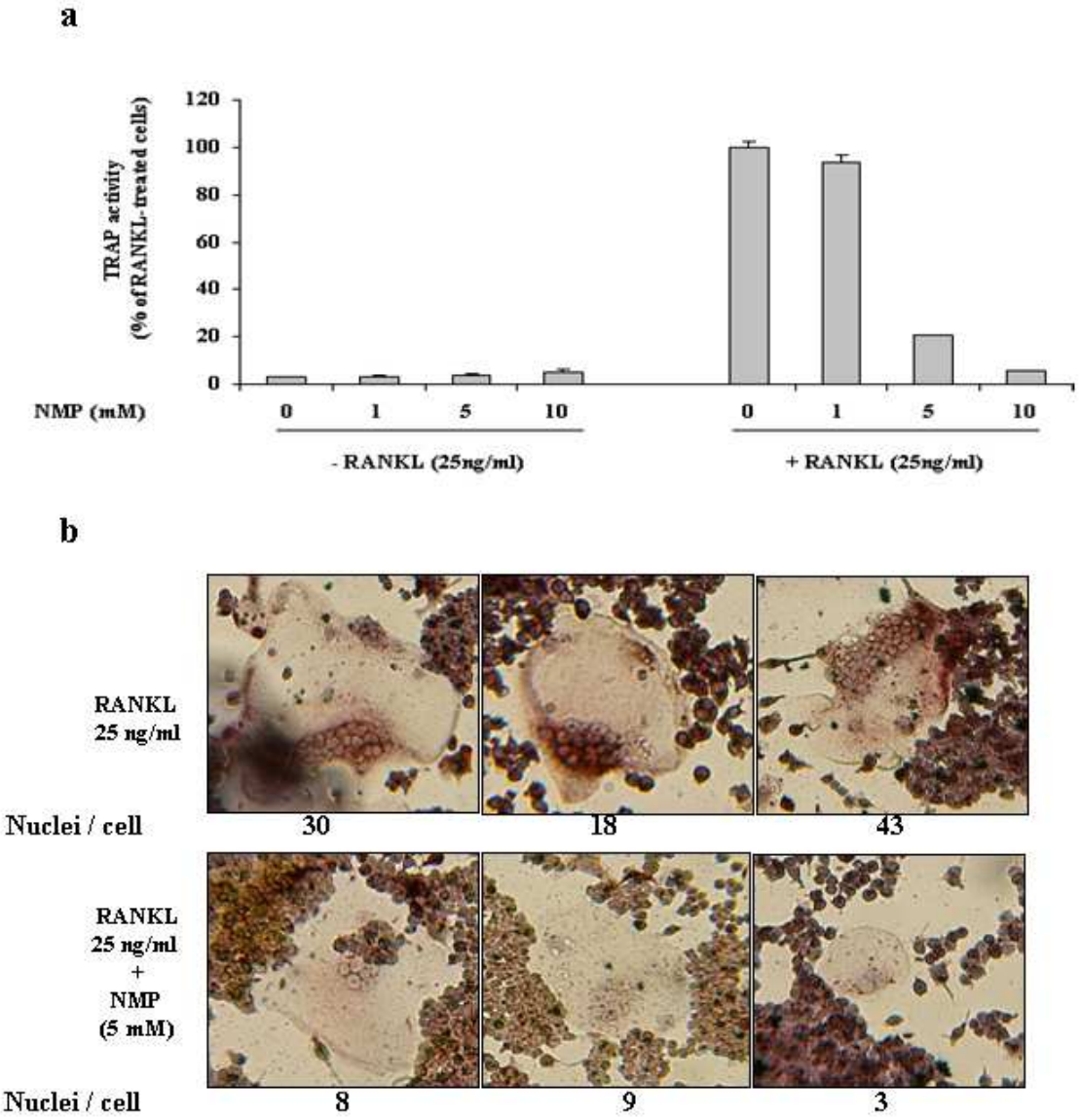

Figure 3. *NMP suppressed osteoclast differentiation and decreased the fusion process.* (**a**) TRAP activity, (**b**) microscopic view of MNCs after TRAP staining. RAW264.7 cells were seeded on a 24-well culture plate, treated with RANKL alone or with different concentration of NMP as indicated in the figure. After 6 days incubation, TRAP activity was measured as described in Methods. Data are expressed as mean S.D. (n=3). In parallel, cells were stained for TRAP after differentiation into mOCs.

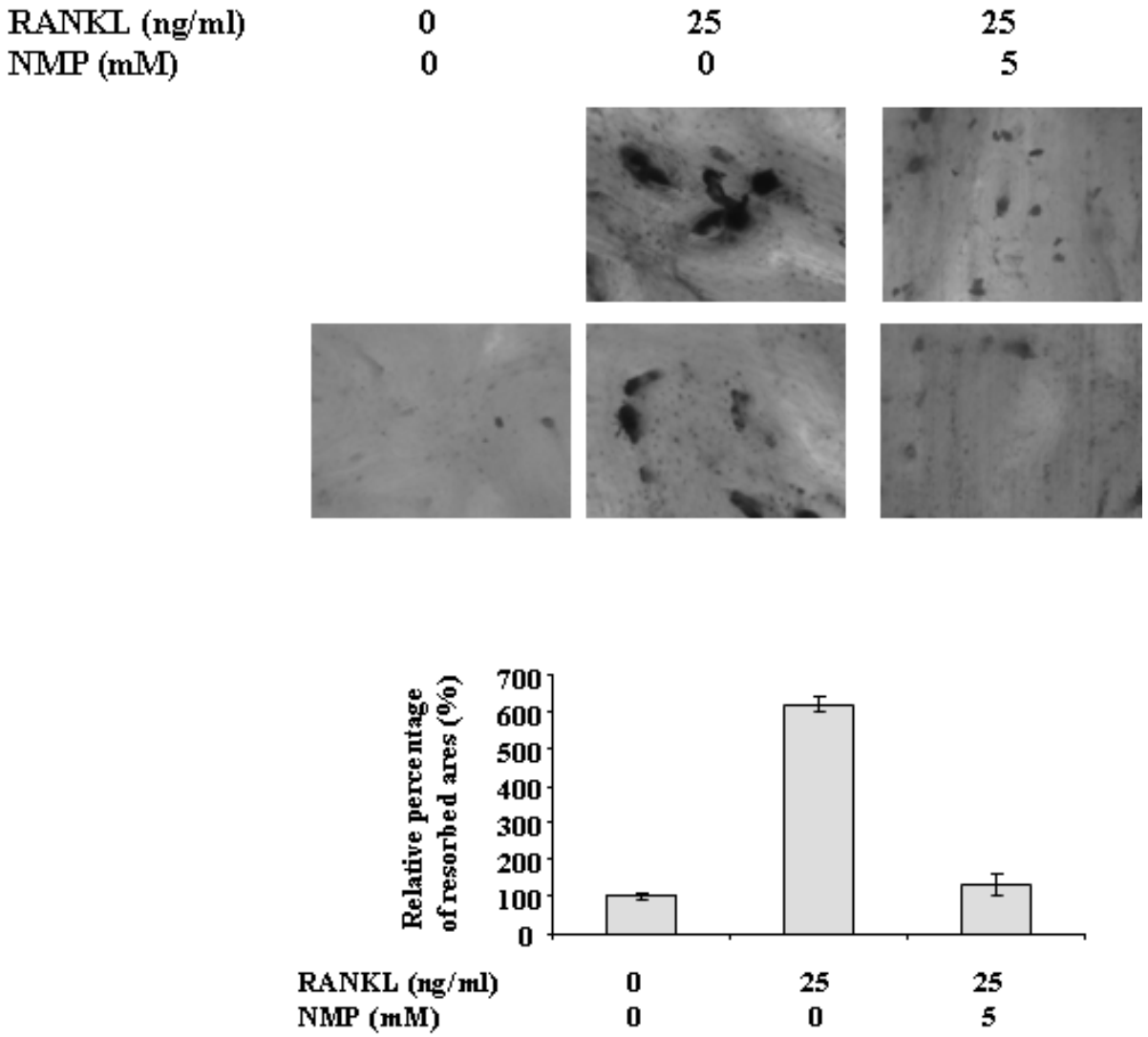

Figure 4. *NMP inhibits RANKL-induced bone resorption.* RAW264.7 cells were cultured on bone slices and treated as indicated on the figure. After 9 days of culture, cells were completely removed and the bone slices were stained with toluidine blue followed by photography (**a**). Histograms represent the percentage of resorbed area (**b**). Data are expressed as mean S.D. (n=3) from a representative experiment.

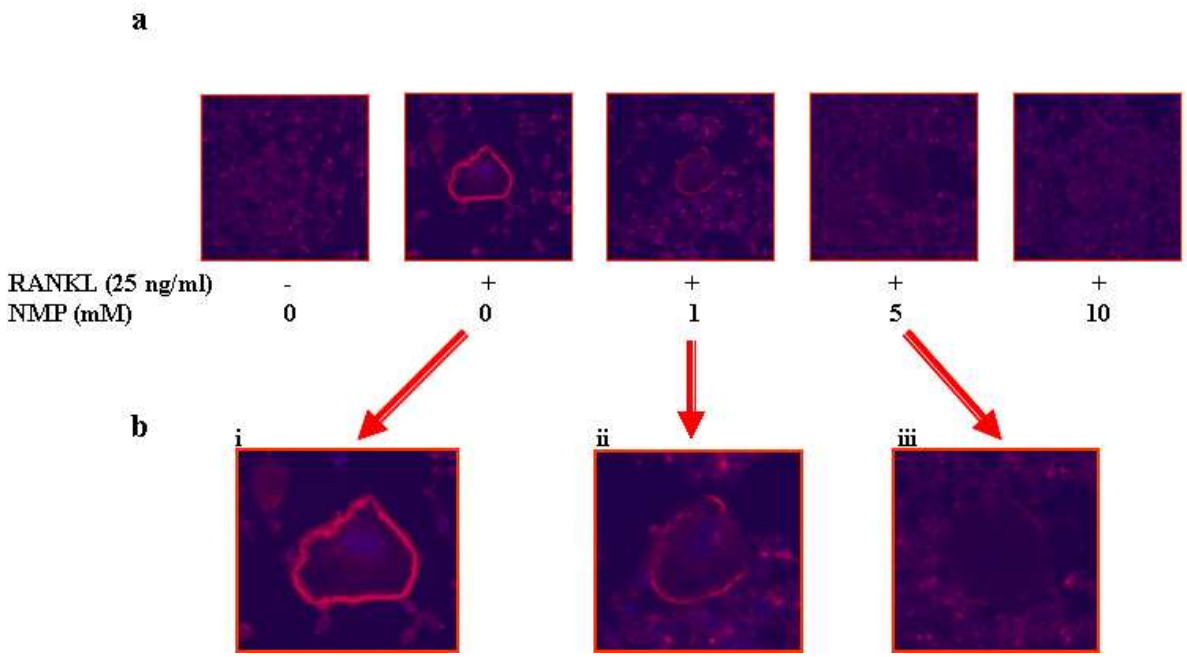

Figure 5. *NMP disrupted RANKL-induced actin rings formation.* RAW 264.7 cells were differentiated into osteoclasts in the presence of RANKL alone or with RANKL and 1, 5 or 10 mM of NMP for 6 days. Cells were fixed and stained for actin rings as indicated in Methods.

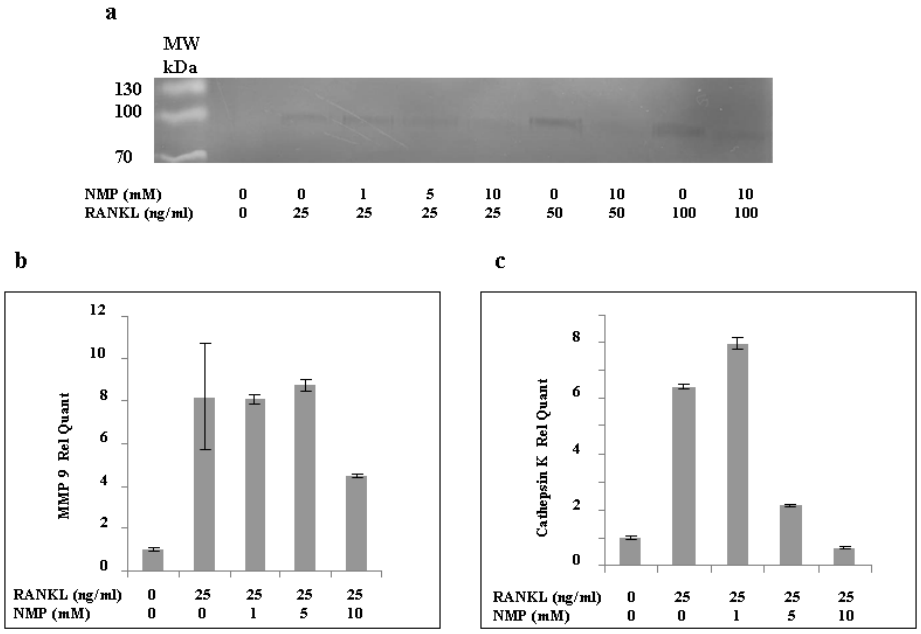

Figure 6. *NMP inhibits RANKL-induced MMP 9 and cathepsin K expression*: **(a)** Representative gelatine zymography assay of MMP-9 activity in RAW264.7 cells. RAW264.7 cells were cultured in serum-free DMEM supplemented with SITE liquid media as indicated in the figure. After 48 hours stimulation aliquots from supernatants were used in zymography assay as described in Methods. **(b and c)**: MMP 9 and cathepsin K mRNAs were determined by real-time PCR after 48h stimulation with RANKL or RANKL in the presence of NMP (as indicated in the figure). Data are expressed as mean S.D. (n=3) from a representative experiment.

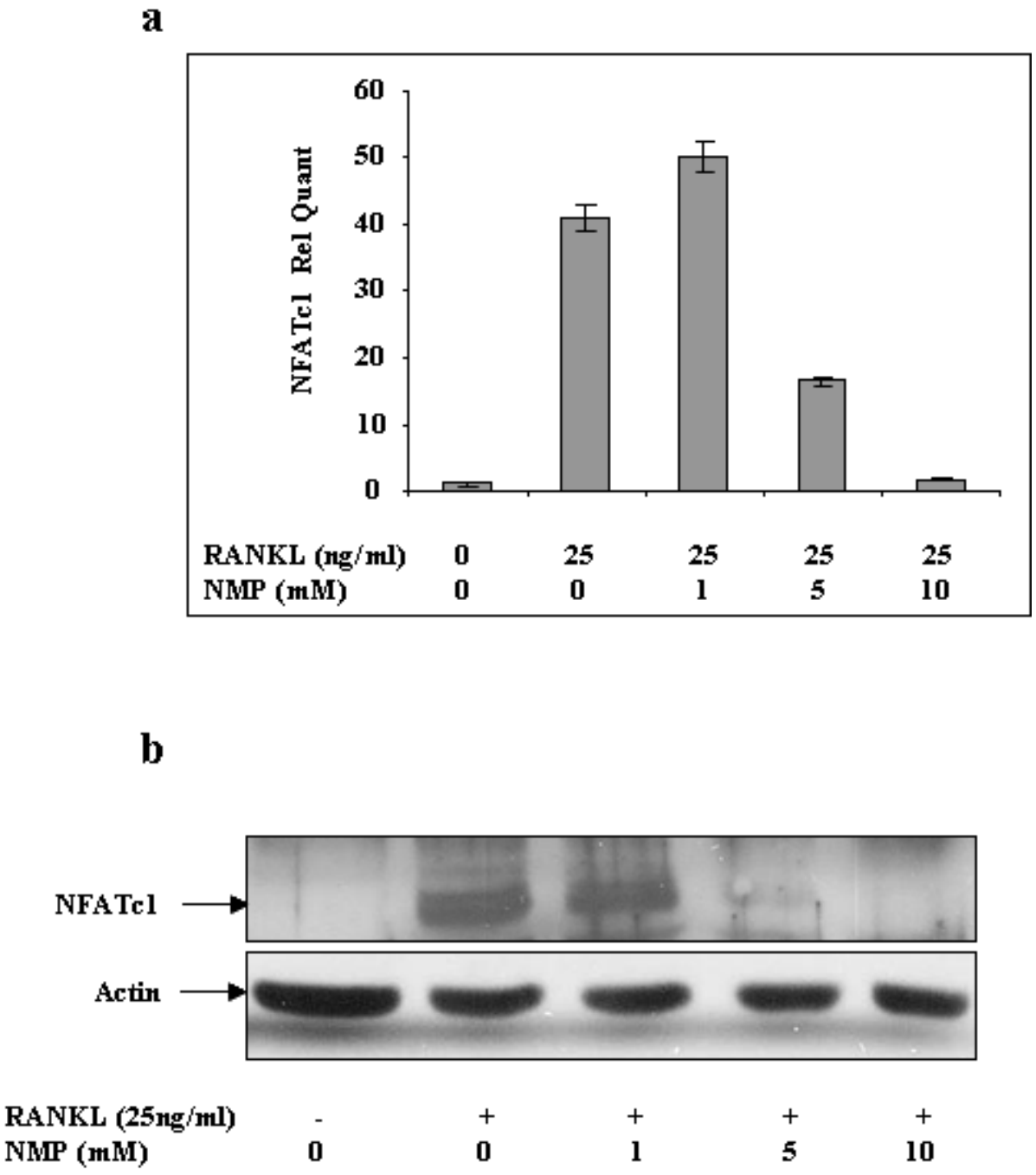

Figure 7. *NMP suppresses RANKL-induced NFATc1 expression.* RAW264 cells were stimulated with RANKL alone or with RANKL and different concentrations of NMP as indicated in the figure (48 h for mRNA and 72 h for proteins). **(a)**: total RNA was extracted and NFATc1 mRNA was determined by real-time PCR as indicated in Methods. Data are expressed as mean S.D. (n=3) from a representative experiment. **(b)**: Whole cell were subjected to Western-blot analysis with antibody against NFATc1. Anti-Actin antibody was used to visualize the loading control.

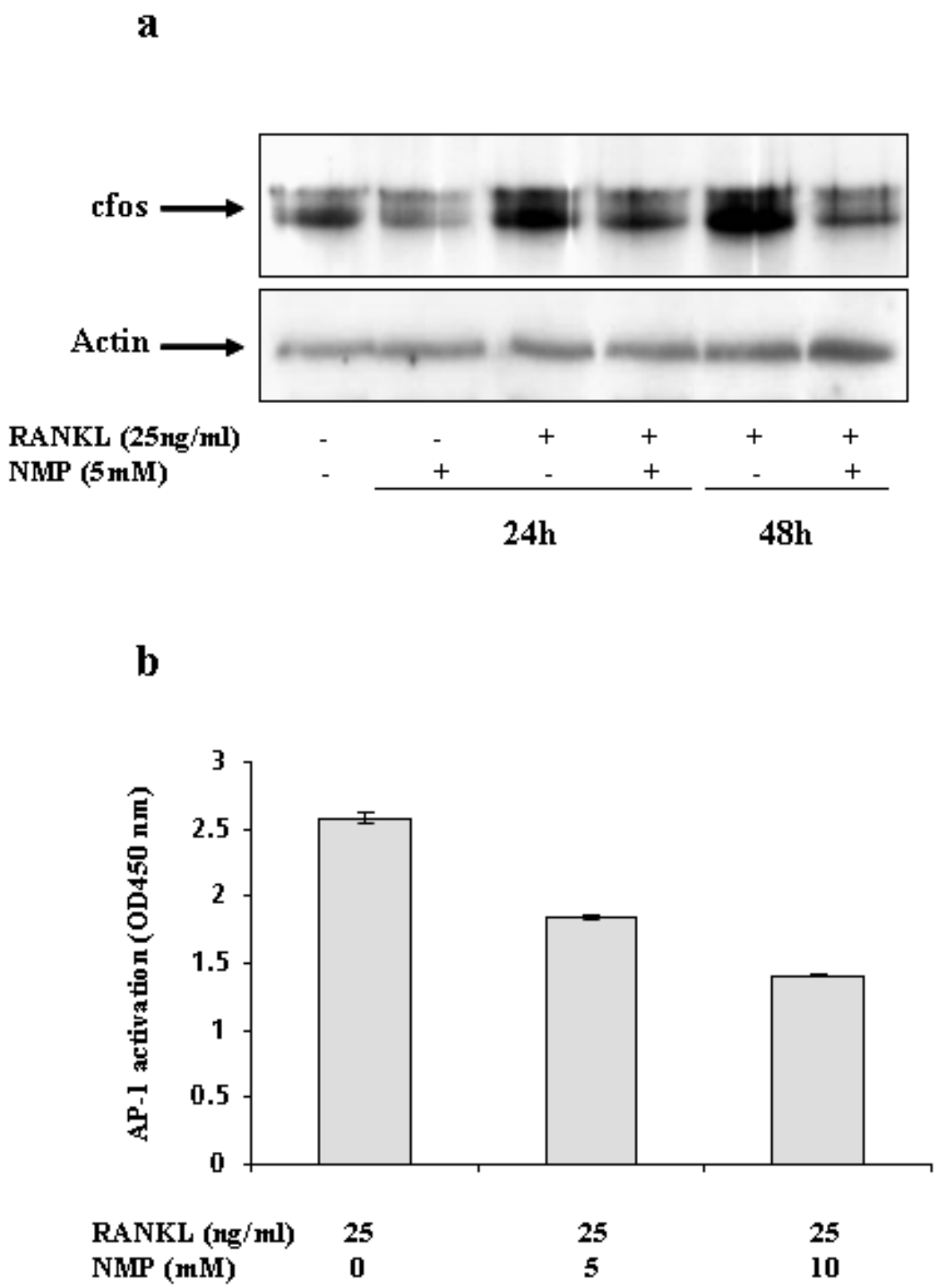

Figure 8. *NMP inhibits RANKL-mediated,C-Fos expression and AP-1 activation.* RAW264.7 cells were treated with RANKL or RANKL and NMP as described in the figure. **(a)** C-Fos protein level was analyzed by western-blot using c-Fos and actin antibodies as indicated in Methods. **(b)** Nuclear extracts from RAW264.7 cells were used to detect AP-1 activation using an ELISA-based method (Trans-AM AP-1, Active Motif, Rixensart, Belgium) as indicated in Methods. The specificity of the assay was monitored by using the K-562 (TPA stimulated) nuclear extract and free wild-type or mutated oligonucleotides according to the manufacturer's instructions. Data are expressed as mean S.D. (n=4) from two different experiments.

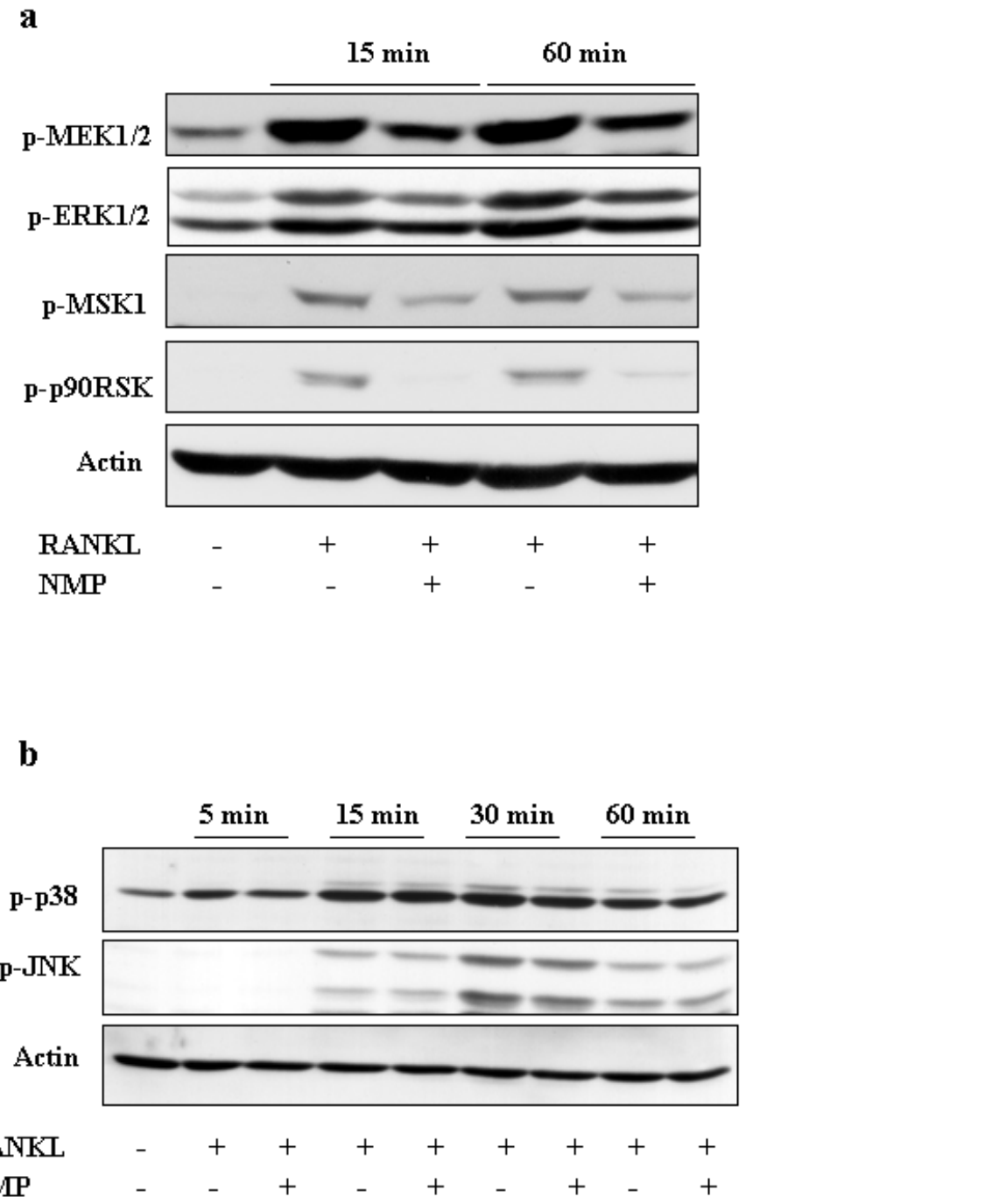

Figure 9. *NMP inhibits RANKL-induced ERK activation but not p38 and JNK activation.* RAW264.7 cells were stimulated as indicated in the figure and whole cell extracts were separated on SDS-PAGE, transferred to PVDF membranes, and probed sequentially with phospho-ERK1/2 pathway antibodies **(a).** RAW264.7 cells were stimulated with RANKL or RANL in the presence of 10 mM of NMP for the indicated time points, and activities of p38 and JNK were examined by Western blot analysis using phosphospecific antibodies **(b)**. Actin antibody was used as loading control.

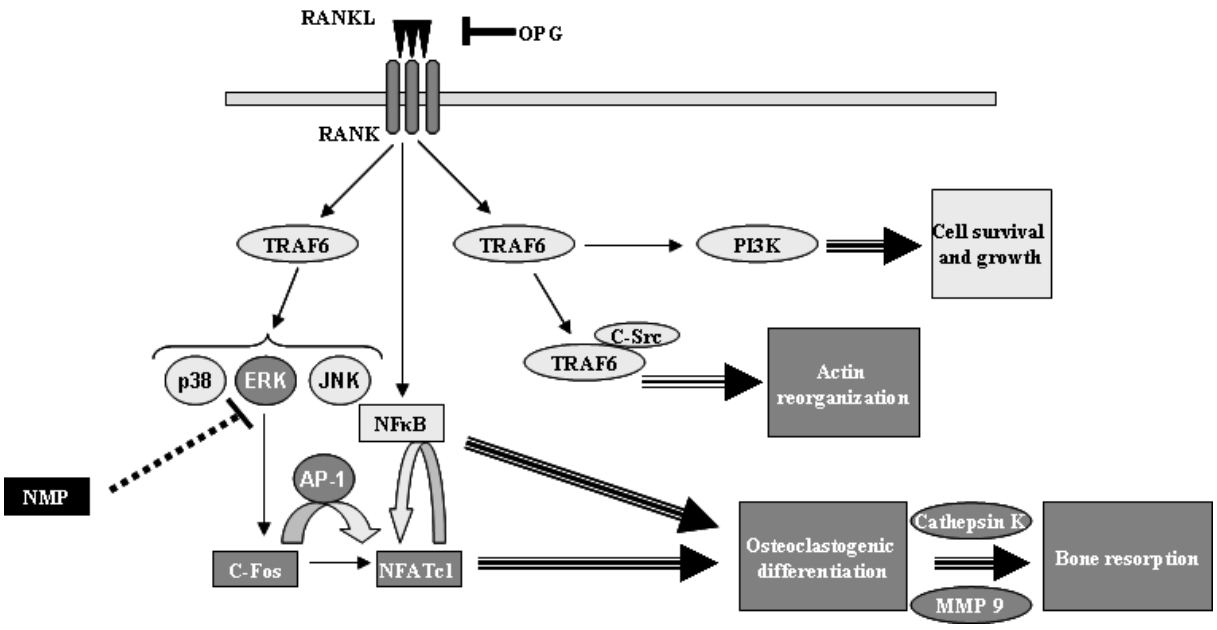

Figure 10. *Schematic model for the osteoclastogenesis inhibitory function of NMP*. RANKL induced the activation of ERK pathway. Inhibition of this pathway by NMP significantly reduces the induction of c-Fos trancription factor and subsequently AP-1 activation by RANKL. The blockade of AP-1 transcription factor considerably decreases NFATc1 trancription factor expression leading to suppresses osteoclastogenic gene expression and subsequent osteoclast formation and function.